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A biosensor for determination of the circulating biomarker CA125/MUC16 by Surface Plasmon Resonance Imaging

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ABSTRACT

CA125/MUC16 is an ovarian tumor cell marker widely used as a biomarker in epithelial ovarian carcinoma. CA125/MUC16 is also used for evaluation of the ROMA (Risk of Ovarian Malignancy Algorithm) value. In this work, a Surface Plasmon Resonance Imaging (SPRI) biosensor for circulating CA125/MUC16 has been developed. The anti-MUC16 antibody was attached to a gold chip via a cysteamine linker. The EDS/NHS protocol was used for the covalent attachment of the antibody. The developed biosensor is specific for CA125/MUC16, and exhibits good recovery and acceptable precision. Its linear response range (2.2–150 U/ml) is well suited to determination of the marker in the blood serum of a healthy control group and, after appropriate dilution, of patients with ovarian cancer. CA125/MUC16 was determined in two series of real samples: blood serum from patients with ovarian cancer and endometrial cysts. The method was validated by parallel determination of the samples using the chemiluminescent Architect i2000 method.

1. Introduction

Carcinoma antigen 125 (CA125), also known as mucin 16 (MUC16), is an ovarian tumor cell marker widely used as a biomarker in epithelial ovarian carcinoma. This is one of the most common types of cancer of the female reproductive system [1]. In terms of structure, the biomarker has been classified as mucin and designated CA125/MUC16 [2]. CA125/MUC16 is a heavily O-glycosylated glycoprotein, which gives it hydrophilic and lubricating properties [3]. It is a component of the female reproductive tract epithelia the respiratory tract and the ocular surface. CA125/MUC16 is a macromolecule consisting of over 22,000 amino acids [3] with masses ranging from about 190 kDa to about 2700 kDa [4]. The presence of isoforms of different sizes may be the reason for variations in MW [4].

CA125/MUC16 has been used as an ovarian cancer biomarker since 1981 [5]. Unlike other markers, the CA125/MUC16 concentration is usually expressed in activity units (U/ml). The marker concentration for ovarian cancer biomarker is reported between 5.4 and 6700 U/ml [1,6–10] and between 4.9 and 88.5 U/ml for healthy donors [1,6,7]. The threshold concentration (cutoff value) above which the result is considered positive is fixed at a value of 35 U/ml [6]. The activities of CA125/MUC16 in the blood serum or plasma of patients with malignant ovarian tumor are on average several times higher than in samples from healthy donors, although particular results represent an unclear picture, with partial overlapping of the results of these two groups. Additionally, the marker concentration for benign ovarian tumor patients is reported between 2 and 2443 U/ml [1,6,7,10]. One of the reasons for the high dispersion of the results may be a shortage of suitable analytical tools for CA125/MUC16 determination. Apart from its use as an ovarian cancer biomarker, CA125/MUC16 is also used for evaluation of the ROMA (Risk of Ovarian Malignancy Algorithm) value, jointly with HE4 [1], and as a biomarker of endometrial cancer [11].

Three analytical methods have recently been diagnostically used for CA125/MUC16 determination: electrochemiluminescence (ECLIA) [1,6,10], Abbot Architect chemiluminescence immunoassay (Architect i2000) [8] and enzyme-linked immunosorbent assay (ELISA) [7]. All three methods are indirect and are based on the formation of sandwich structures with two antibodies. To create an analytical signal, the outside antibody is linked to horseradish peroxidase (Architect i2000 and ELISA) or a ruthenium complex (tris(2,2′-bipyridyl) ruthenium(II) complex, $Ru(bpy)_3$) (ECLIA). Additionally, ECLIA and Architect i2000 include preconcentration of the marker on magnetic beads. However, numerous new proposals have been published. The most recent of them proposed microfluidic systems with functionalized gold nanoparticles [12] or functionalized carbon nanotubes [13] containing immobilized antiCA125 antibody and a capacitive signal measurement.

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Accumulation with a magnetic nanoparticle–antiCA125 antibody conjugate prior to final determination by fluorescence is also proposed [14]. Papers concerning CA125 determination are analyzed in a review by Razmi and Hasanzadeh [15].

Surface Plasmon Resonance Imaging (SPRI) is an emerging, direct, "label-free" technique having growing potential in the development of diagnostic biosensors. An advantage of the method is the relatively simple construction of the biosensor. It consists of a linker attached to a gold surface and a receptor (antibody or inhibitor). The biomarker to be determined is entrapped from the solution (diluted blood serum, plasma or urine). Two operational solutions are employed for measurement: fluidic and stationary (non-fluidic). Fluidic measurement is usually performed with *in situ* creation of a biosensor, while in the non-fluidic case the biosensor is prepared *ex situ*. In the fluidic version a biosensor is created during the measurement by sequential introduction of a linker, a receptor and a solution containing the determined marker. Measurement is performed with the biosensor in contact with the solution. This is the major difference from the non-fluidic case. Non-fluidic measurement is usually performed in a stationary arrangement, with an array of separated measuring points. An array of measuring points is used for a single measurement to improve the precision of the result. Applying the stationary SPRi version in a model investigation [16] and in the determination of different biomarkers in real clinical samples [17–19] it has been demonstrated that this technique is suitable for use without preliminary analyte preconcentration or signal enhancement. The growing number of clinical applications of SPRi is shown in a recent review [20].

The aim of this work was to develop an SPRi biosensor and a method for the determination of CA125/MUC16 suitable for determination of the biomarker in blood serum or plasma, and to validate the developed biosensor and method.

2. Materials

2.1. Reagents

Recombinat Human CA-125/MUC 16 (5609-MU) from Bio-techne, Poland, rabbit polyclonal Anti- MUC16 antibody (ab133419) from Abcam, Germany, interleukin 6 (I1395) from Aldrich, Munich, Germany, albumin (A2153) from MERC, Poland, leptin (ab51273) from Abcam, Germany, metalloproteinase-2 (SRP3118) from MERC, Poland, cysteamine hydrochloride, N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma Steinheim, Germany), N-Hydroxysuccinimide (NHS) (Aldrich, Munich, Germany) were used. HBS-ES solution pH = 7.4 (0.01 M HEPES, 0.15 M sodium chloride, 0.005% Tween 20, 3 mM EDTA), photopolimer ELPEMER SD 2054, hydrophobic protective paint SD 2368 UV SG-DG (Peters, Kempen, Germany, www.peters.de/), Phosphate Buffered Saline (PBS) pH = 7.4, carbonate buffer pH = 8.5 (BIOMED, Lublin, Poland) were used as received. PBS buffer was used for dissolution and dilution of the antibody and proteins. Aqueous solutions were prepared with MilliQ water (Simplicity®MILLIPORE).

2.2. Biological samples

Blood samples were obtained from the cubital vein. Serum was prepared according to standard procedures. Serum samples were frozen immediately and kept at −80 °C. Depending on the concentration of the CA125/MUC16 marker in biological samples determined using the standard method, twofold and tenfold dilutions were made with phosphate buffered saline (PBS). Serum samples from patients with ovarian cancer and endometrial cysts were collected before surgery. Approval for this study was obtained from the Bioethics Committee of the Medical University of Bialystok (Bialystok, Poland) and written informed consent was obtained from all patients.

2.3. Chip preparation

Gold chips were manufactured as described in previous papers [21,22]. The gold surface of the chip was covered with a photopolymer and hydrophobic paint, a procedure described previously [21,22]. As a result, 9 × 12 free gold surfaces were obtained. With the use of this chip, nine different solutions can be simultaneously measured without mixing the tested solutions, and twelve single SPRi measurements can be performed from one solution.

2.4. Receptor (antibody) immobilization

Chips were rinsed with ethanol and water and dried under a stream of argon. They were then immersed in 20 mM ethanolic solution of cysteamine for at least 2 h, after which they were again rinsed with ethanol and water and dried under a stream of argon. The next step was the immobilization of the receptor (antibody). Receptor (antibody) solution in a PBS buffer (200 μg/ml) was activated with NHS (50 mM) and EDC (200 mM) in a carbonate buffer (pH = 8.5) environment. Then the activated receptor (antibody) was placed on a thiol (cysteamine) modified surface and incubated at 37 °C for 1 h.

3. Methods

3.1. SPRi measurements

SPRI measurements for the protein biosensor array were performed as described previously [21,22]. The signal was measured twice on the basis of registered images, after the immobilization of the receptor (antibody) and then after interaction with CA125/MUC16. Serum samples were placed directly on the prepared biosensor for 10 min to allow interaction with the receptor (antibody). The volume of the sample applied to each measuring field was 3 μl. After this time the biosensor was washed with water to remove unbound molecules from the surface. SPRi measurements were performed at a constant angle of light. Two images were recorded: the first image reflects the immobilization of the receptor (antibody) (see Fig. 1S), and the second image shows the interaction of CA125/MUC16 with the sample containing the analyte (see Fig. 2S). Non-specific binding was monitored by measuring the SPRI signal in place on the chip without the receptor (ligand). Non-specific binding was minimized by preparing samples in a PBS buffer and by placing BSA in PBS buffer onto the chip. The SPRI signal, which was proportional to the mass of entrapped CA125/MUC16, was obtained as the difference between the signals before and after interaction with the analyzed sample, for each spot separately.

3.2. Standard method used for validation of the developed biosensor

The standard method used to determine the CA125/MUC16 marker was the Abbot Architect chemiluminescence immunoassay test (Architect CA125 II). The determination was performed by a routine diagnostic laboratory. The method is indirect and is based on the formation of sandwich structures with two receptors (antibodies) [23]. To create an analytical signal, the outside receptor (antibody) is linked to horseradish peroxidase. Chemiluminescence is used as the detection method. Additionally, the method includes preconcentration of CA125/MUC16 on magnetic beads.

4. Results and discussion

4.1. Optimization of receptor (antibody) concentration

The purpose of this investigation was to find the best conditions for the determination of CA125 concentration. The investigation was performed using the receptor (antibody) in a concentration range of 10–350 μg/ml at a constant activity of CA125 (31.2 U/ml). For this

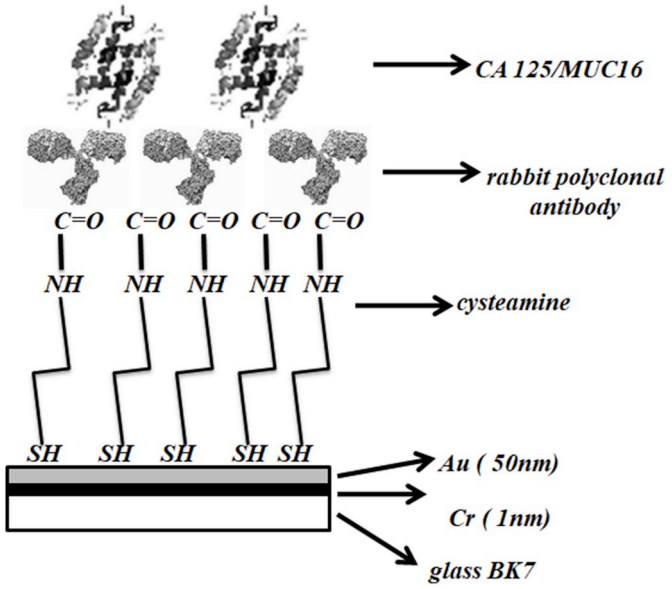

Fig. 1. Diagram of the active part of the sensor.

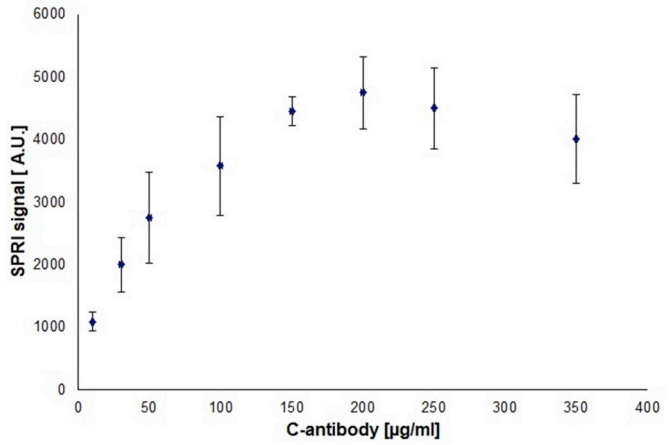

Fig. 2. Dependence of SPRI signal (Arbitrary Units) on rabbit anti-human CA125/MUC16 antibody concentration. CA125/MUC16 activity: 31.2 U/ml. pH = 7.4. Error bars were calculated for 12 independent measurements for each concentration at a 95% confidence level.

purpose, eight antibody solutions with concentrations 10, 30, 50, 100, 150, 200, 250, 300 µg/ml were used. Briefly, after preparation of a chip with the specific receptor (antibody) and CA125 binding, the SPRI measurement was performed. The results are given in Fig. 2. The obtained curve is of the Langmuir isotherm type. The plateau of the signal is observed for receptor (antibody) concentrations above 200 µg/ml, and this concentration was selected for further experiments.

4.2. Calibration curve for CA125/MUC16

The calibration curve for CA125/MUC16 determination with a biosensor consisting of cysteamine linker and the receptor (antibody) as the receptor (antibody) was obtained in optimized conditions. SPRI measurements were performed in a range between 1.04 and 208 U/ml. The results are shown in Fig. 3. The curve is of typical Langmuirian type. The plateau of the curve corresponds to the saturation of active sites of the biosensor. The initial section of the curve is linear and is useful for analytical purposes. It covers the concentration range from the limit of quantification to above 150 U/ml.

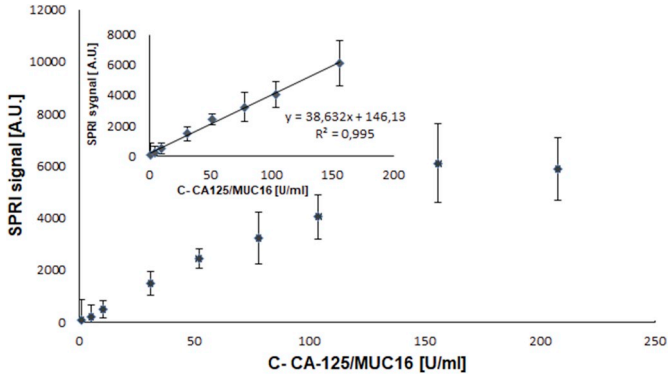

Fig. 3. Dependence of the SPRI signal (Arbitrary Units) on CA125/MUC16 concentration. Rabbit anti-human CA125/MUC16 antibody concentration: 200 µg/ml pH = 7.4. Error bars were calculated for 12 independent measurements for each concentration at a 95% confidence level.

4.3. Selectivity of the biosensor

In order to demonstrate the selectivity of the biosensor, the interferences of different compounds to the CA125 response were examined. Albumin, leptin, interleukin 6 and metalloproteinase-2 were used as potential interferents. This selection of potential interferents is based on literature reports and the specifications of commercial ELISAs used to determine these substances. Albumin is present in blood at a very high level. Leptin is reported to be a marker useful in the diagnosis of ovarian cancer [24,25], as is metalloproteinase-2 [26]. Interleukin 6 is considered to be a marker of endometriosis and ovarian cancer [27,28].

The specificity of the interaction between receptor (antibody) and CA125 was verified by treating a surface of the chip with immobilized receptor (antibody) layer (200 µg/ml) with mixtures of CA125 and potential interferents. Mixtures with different ratios of CA125 (10.4 U/ml) and the interfering proteins were tested. The results are shown in Fig. 4. Albumin, leptin, interleukin 6 and metaloproteinase-2 were found to have no influence on the results of determination of CA125 concentration. Thus, the high selectivity of the biosensor was confirmed.

4.3.1. Precision and recovery of the method

Three series of measurements with respective CA125 spikes of 1.04, 52 and 104 U/ml were performed for the determination of recovery, precision, limit of detection (LOD) and limit of quantification (LOQ). The results are shown in Table 1.

Both precision and recovery are typical for this kind of

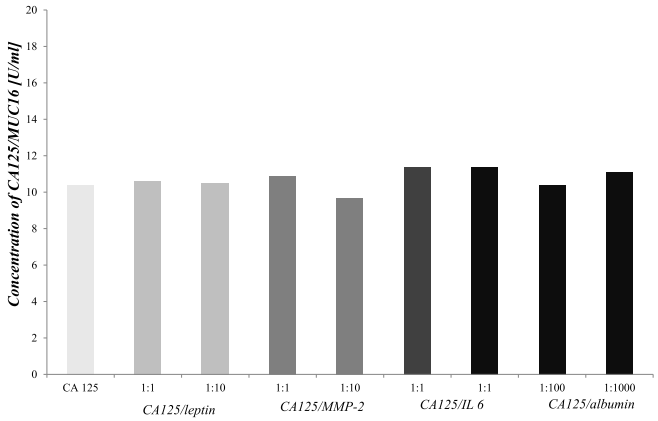

Fig. 4. Influence of human albumin, leptin, interleukin-6, and metaloproteinase-2 on the determination of CA125/MUC16 concentration (10.4 U/ml) by the SPRI method.

Table 1
Recovery and precision of CA125/MUC16 determination.

Added [U/ml]	Found [U/ml]	SD [U/ml]	Recovery [%]
1.04	1.3	0.22	128
52	51	20.3	97
104	105	12.6	101

Table 2
CA125 activity in serum samples of ovarian cancer and endometrial cyst patients as determined by the newly developed and standard methods.

Number of samples	Patients with ovarian cancer activity of CA 125 [U/ml]		Patients with endometrial cysts activity of CA 125 [U/ml]	
	SPRI metod	Standard method	SPRI method	Standard method
1	178.4	266.6	73.5	79.8
2	1061.1	> 1001	27.3	31.6
3	524.5	> 1001	42.3	41.6
4	115.5	176.9	22.0	42.0
5	426.4	> 1001	18.9	18.9
6	266.6	220.2	34.4	56.0
7			77.5	36.4
8			33.4	33.2
9			21.1	36.2
Average	428.8	611.1	38.9	52.9

determination, and they are satisfactory. The recoveries of CA125 spikes were found to lie in the range 99.9–135%. The detection limit, calculated on the basis of three standard deviations (SDs), is equal to 0.66 U/ml. Thus, the limit of quantification (10 SDs of the blank) is equal to 2.2 U/ml.

4.4. Determination of CA125/MUC16 concentration in biological samples

The concentration of CA125/MUC16 in biological samples was determined using the developed SPRI biosensor. The tests were performed on nine samples of serum from patients with endometriosis and from patients with ovarian cancer. The aim of this experiment was to verify the suitability of the developed method for the analysis of typical real samples. The determination was performed as described in previous sections. The CA125/MUC16 concentration was evaluated on the basis of a calibration curve. The same samples were determined in a routine diagnostic laboratory using the chemiluminescence immunoassay test Architect CA125 II. The results are shown in Table 2.

The results of the two methods show acceptable agreement for the samples from patients with endometrial cysts. In the case of samples from patients with ovarian cancer, approximately half of the results exhibit agreement, while for the remainder, the newly developed method returns lower results than the standard method. It should be noted that half of the ovarian cancer samples were not precisely determined by the Architect CA125 II test, lying above the range of that method.

The newly developed method is well suited to the determination of CA125/MUC16 in blood serum samples. The linear response range extends from 2.2 (LOQ) to 150 U/ml. Samples are diluted with PBS buffer before measurement. Thus, the whole of the ranges for malignant [1,6–10] and benign [1,6,7] ovarian tumor and healthy donors [1,6,7] (2–6700 U/ml) can be covered. No significant interference was observed (Fig. 4), as was confirmed by the determination of CA125/MUC16 in the series of serum sample measurements (see Table 2). Any significant interference from the serum matrix would have considerably altered the results. The recoveries of CA125/MUC16 determination (Table 1) are satisfactory, taking into account that the experiment at 1.04 U/ml was performed on the edge of the range of applicability of the method (above LOD but below LOQ). The precision of determination by the developed method is only acceptable, but is typical for this kind of measurements. The precision of final determination may be improved by performing a short series (3–5) of measurements of the same sample. It should be noted that only a 3 µl volume of serum is required for a single measurement, and the developed biosensor enables the simultaneous determination of nine samples. A comparative investigation of the developed method and the standard chemiluminescence method with respect to samples from patients with endometrial cysts and patients with ovarian cancer showed partial agreement of the results (Table 2). Some of the results obtained by the developed method are lower than those for the standard method, but are closer to the median values reported in the literature [1,6–10].

In terms of complexity, the newly developed method is much simpler than the other applied methods. It merely requires immobilization of the rabbit anti-human CA125/MUC16 receptor (antibody) via the cysteamine linker and entrapment of CA125/MUC16 from blood serum before SPRi measurement. The standard chemiluminescence method, which was used for comparison with the developed method, includes the preconcentration of CA125/MUC16 on magnetic functionalized beads, the formation of a sandwich structure with the second receptor (antibody) conjugated with horseradish peroxidase, and the creation of chemiluminescence in a catalytic reaction [23]. The greater number of steps entails more sources of error. It should be noted that the first stage of the chemiluminescence method is similar to the whole of the CA125/MUC16 entrapment process in the developed SPRi method. ECLIA and ELISA are equally complex multistage procedures.

5. Conclusions

An SPRi biosensor has been developed for determination of the circulating biomarker CA125/MUC16. It is specific for CA125/MUC16, and exhibits good recovery and acceptable precision. The linear range of the biosensor's response is well matched to the CA125/MUC16 concentrations found in the blood of healthy donors and of patients with ovarian cancer (after dilution). The biosensor is competitive with respect to currently applied methods (ECLIA, Architect i2000 and ELISA). It is characterized by much simpler construction and operation than the other methods used for CA125/MUC16 determination.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.120187.

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